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Role of Ets-2 in tumor-associated macrophages during breast cancer progression

Tahera Zabuawala

(Investigator-Initiated Research Award Recipient)

Abstract

While it is known that the most common human tumors are derived from epithelial cells that have undergone multiple genetic alterations, it is also becoming clear that the alterations in the tumor micro-environment are necessary for tumor progression. One such stromal component is the macrophage. Recent studies have shown that deletion of CSF-1, an essential growth factor for growth and differentiation of macrophages, delays pulmonary metastasis in the PyMT breast cancer model in mice. Previous work from our lab has demonstrated that Ets-2 is a nuclear effector of the Ras-Raf-MAP kinase pathway. My hypothesis is that CSF-1 mediates its pro-tumorigenic effects in macrophages via activation of Ets-2.

To test this hypothesis, my project aims to analyze the effects of Ets-2 deletion specifically in the tumor-associated macrophages (TAMs) in the breast tumor microenvironment. To achieve this, I am using a conditional Ets-2 "floxed" allele available in our lab. I am using a non-inducible Lys-Cre transgene to delete Ets-2 specifically in the macrophages. Preliminary results with this system indicate that the gross tumor volume in the experimental animals is similar to that of the controls. Interestingly, the area of the lung lesions is significantly less in the experimentals as compared to those of the controls. At present I am trying to determine whether it is the exit from the primary tumor site or growth in the lungs which is affected in the experimentals.

Microarray and real-time PCR analysis of mammary TAMs indicate that anti-angiogenic factors may be downregulated in the Ets-2 deleted TAMs.

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Role of Ets-2 in tumor-associated macrophages during breast cancer progression

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Introduction

Every two minutes a woman in the United States is diagnosed with breast cancer. Yet, the exact cause of cancer is unknown. To find therapeutic drugs to fight cancer, it is crucial to understand the crosstalk going on between different genes in the different cellular compartments of the tumor micro-environment. I propose to dissect the role of one of the genes called Ets-2 in the macrophage compartment of the tumor micro-environment. Preliminary data indicated that in-vivo deletion of ets-2 in the tumor associated macrophages (TAMs) decreased the incidence of lung metastasis in a spontaneous murine breast tumor model (PyMT, Polyoma Virus Middle T antigen). The aim of this training grant is to study the mechanism of the observed phenotype by various techniques like micro-array, histology etc. During the course of the study, one of the reagents, Lys-Cre transgenic mice, were shown to inefficiently detete ets-2 in TAMs. So another aim is to develop other transgenic mouse reagents to better delete ets-2 in the mouse macrophage compartment.

Body

Task I: To study the mechanism of metastasis in 'macrophage' ets-2 deleted females.

As mentioned earlier, preliminary studies indicated that deleting ets-2 in the macrophage compartment of the tumor micro-environment cause smaller lung metastatic lesions while having no effects in the primary tumor progression. However, it is not yet clear whether *ets-2* is important for the exit of tumor cells from the breast tissue or for their entry into the lungs. To test whether latter is the case, tail-vein injections using a PyMT cell line, Met-1 (obtained from Cardiff lab, University of California- Davis), were performed. In collaboration with Rosol lab (The Ohio State University), the Met-1 cell line was tagged with a luciferase reporter gene. This would enable better analysis of lung metastasis (by using IVIS imaging equipment available on campus). However, unfortunately, even after using several different approaches to transfect the cells, a stably expressing Met-l-luciferase cell line could not established. At present, another mouse breast tumor cell line, MVT-1 (obtained from Johnson lab, Georgetown university), were obtained. We are now trying to tag these cells to a luciferase reporter gene using a retro-viral approach of transfection.

Task II: To analyze various tumor-associated macrophages, fibroblasts and epithelial cells when ets-2 is deleted in the macrophages

In this study, when ets-2 is genetically deleted in the macrophage compartment of the tumor micro-environment (TME), a phenotype in the epithelial (tumor) cells is observed. This alludes to the fact that due to 'macrophage' ets-2 deletion, there may be changes occurring in the TAMs that affect the tumor cell behavior. To test this hypothesis, a

Transgenic mouse strain expressing YFP in the macrophages was developed. YFP positive macrophages were extracted from tumor-bearing mice at different stages by FACS (Flourescent Activated Cell sorting) analysis. Global gene expression levels were analyzed by micro-array profiling. A number of genes were upregulated

when 'macrophage' ets-2 was deleted. The different gene-expression trends are shown in Figure1. As seen in Trend-III in Figure 1, a group of genes had opposite effect in wild-type and ets-2 deleted TAMs. Some of these genes have been reported to have an anti-angiogenic and metastasis-suppressing effect.

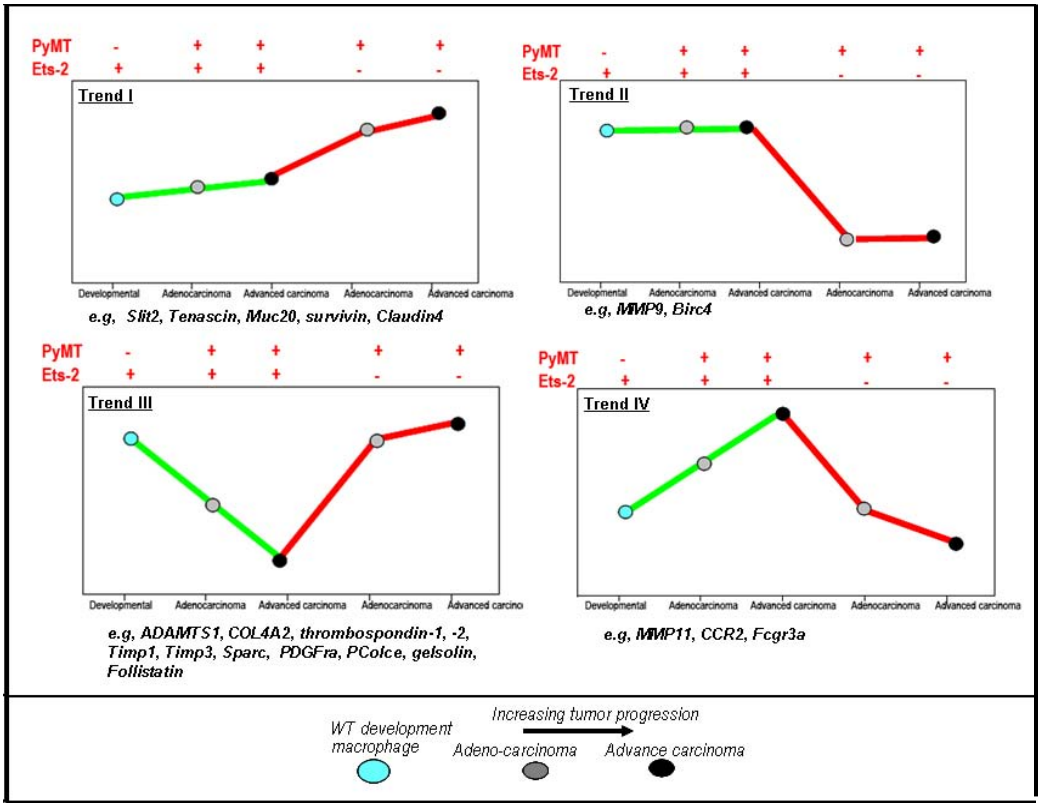

Figure 1: Analysis of various trends observed in gene-expression patterns in wild-type and ets-2 deleted Tumor Associated Macrophages (TAMs). Solid green line re presents trend observed in wild-type ets-2 macrophage and red represnts that in ets-2 deleted macrophage

The anti-angiogenic genes, like Timp1, ADAMTS-1, THBS-1, and THBS-2 were upregulated in ets-2 deleted TAMs and further confirmed by real-time PCR analysis (Figure 2)[1, 2]. Angiogenesis is a process of sprouting of new blood vessels from existing ones. Since tumor cells, like normal cells, also require oxygen and nutrients for their growth, there is increased angiogenesis during tumor progression. Anti-angiogenic genes 'switch-off' this angiogenic switch. Thus the micro-array data indicates that ets-2 may act as a repressor of anti-angiogenic genes during tumor progression.

Recent studies have shown that a set of genes termed 'metastasis suppressors' inhibit metastasis while having little effect on primary tumor progression[3, 4]. Our micro-array analysis indicated that these genes were upregulated in 'ets-2 deleted' TAMs (Figure 2). Ets-2 might be repressor of metastasis-suppressors during tumor progression.

The promoter sequence of some of the anti-angiogenic and metastasis suppressor genes were analyzed for conserved ets binding sites. Some of the genes like Timp-1, SPARC etc have conserved sites (Figure 3a). To test whether ets-2 binds to the promoter of these genes, a ChIP (Chromatin Immuno-precipitation) was performed on cultured bone marrow macrophages. As shown in Figure 3b, ets-2 does bind to these promoters in-vitro. From FACs analysis we have found that TAMs may represent less than 5% of the tumor cell extract. At present, efforts are underway to develop a method to analyze promoters of these 'repressed' genes in TAMs in-vivo.

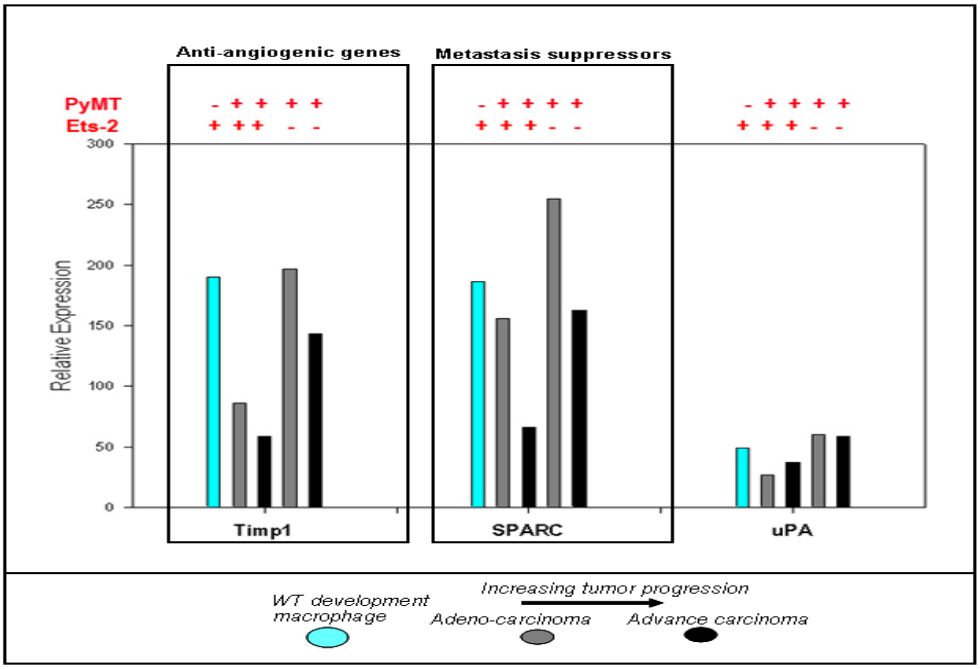

Figure 2: Real-time PCR confirmation of some of the ant-angiogenic and metastasis suppressor genes affected by deletion of 'macrophage' ets-2 in the tumor micro-environment (TME). Some genes like uPA that have been shown to be ets-2 targets in the macrophages in-vitro are not affected

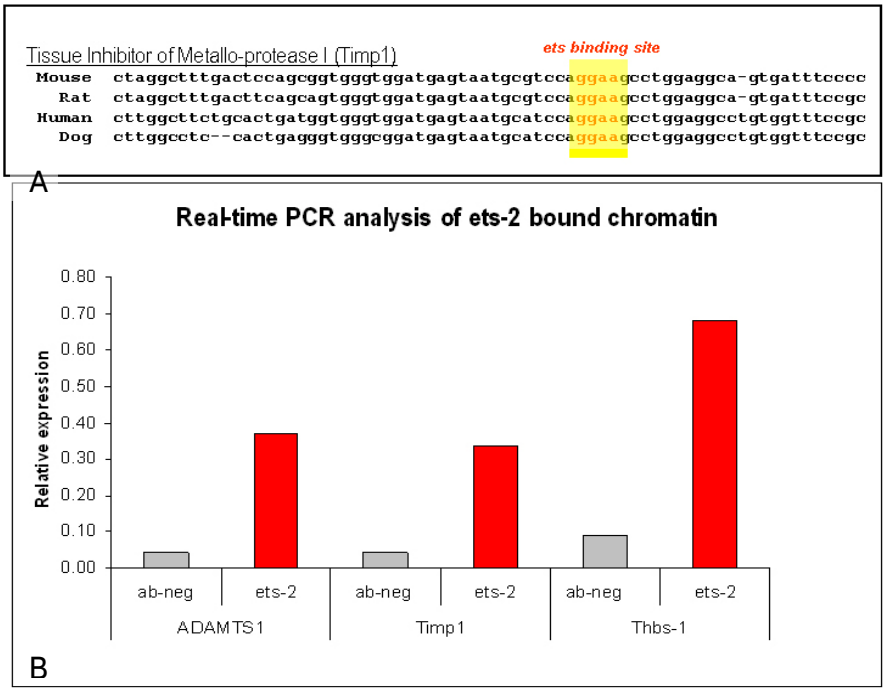

Figure 3: A. Promoter sequence analysis of ets binding site of an anti-angiogenic gene, Timp1. the conserved ets binding site is highlighted in yellow. B. Chromatin Immuno-precipitation (ChIP) analysis of some of the anti-angiogenic genes in cultured wild-type bone-marrow macrophages. The ets-2 pulldown is compared to antibody-negative (ab-neg) sample wherein no antibody is added during the ChIP procedure

Apart from biochemical assays, histological studies were performed on the metastatic lung tissue sections of tumor bearing mice. Interestingly, we observed that when ets-2 is deleted, there is a difference in macrophage localization in the lung lesions (Figure 4). At present, I am analyzing the expression of the above-mentioned 'repressed' genes in the lung lesions by immuno-histochemical methods.

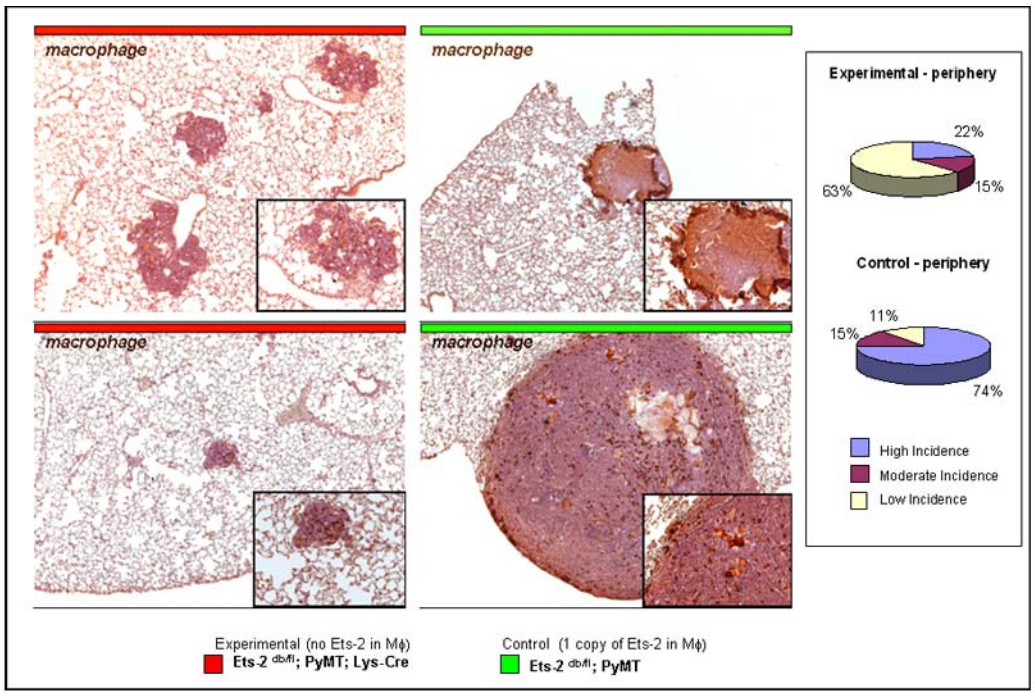

Figure 4: Immuno-histochemical analysis for macrophages distribution in the lung lesions. Rat α–mouse F4/80 antibody was used as a marker of macrophage (stained brown). The lung lesions were qualitatively analyzed and scored for peripheral macrophage distribution (pie-chart shown in right)

TaskIII: To extensively characterize the c-fms-rtTA-M2 and c-fms-rtTA-S2 founders

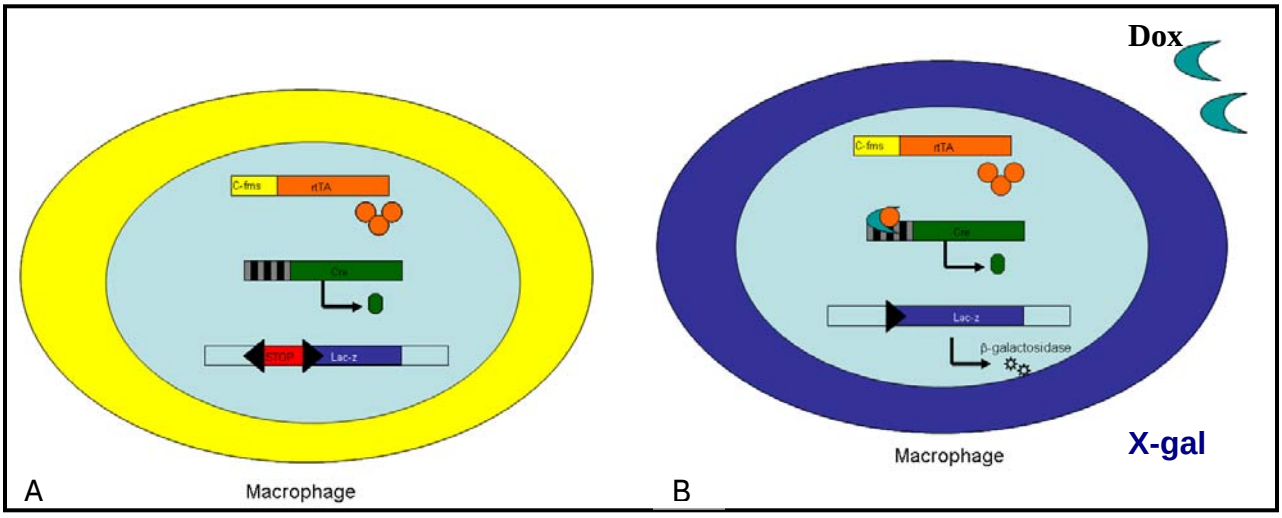

Figure 5: Schematic representation of reporter gene analysis adopted to test the various c-fms-rtTA-M2 and –S2 transgenic lines. Briefly, in the absence of Doxicycline (Dox), the expression of beta-galactosidase (encoded by lac-z gene) is not induced. If rtTA is expressed strictly in the macrophages, then in the presence of Dox (B), beta-galactosidase is induced. The enzymatic activity can be assayed by its ability to convert a colorless substrate, X-gal, to a blue colored residue

To test whether the expression of the rtTA protein in the *c-fms*-rtTA-M2 and –S2 transgenic mice was expressed in macrophages under Dox conditions only, a reporter gene analysis study was performed. In this study c-fms-rtTA-M2 and –S2 lines [5] were bred with mice carrying conditional Rosa-LoxP reporter allele and Tet-O-Cre allele (Figure 5). Beta-galactosidase assay was performed on various tissue sections extracted from Dox fed c-fms-rtTA; Tet-O-Cre; Rosa-LoxP mice. One of the –M2 lines seem to express rtTA in

macrophages under Dox (Figure 6c). I have further characterized this founder line. As seen in Figure 6a, in mammary gland, this transgene is expressed in the macrophages under Dox conditions only. When a conditional ets-2 allele is used, ets-2 is deleted in cultured bone marrow cells only when Dox is added.

At present, I am characterizing this strain during various stages of embryo development.

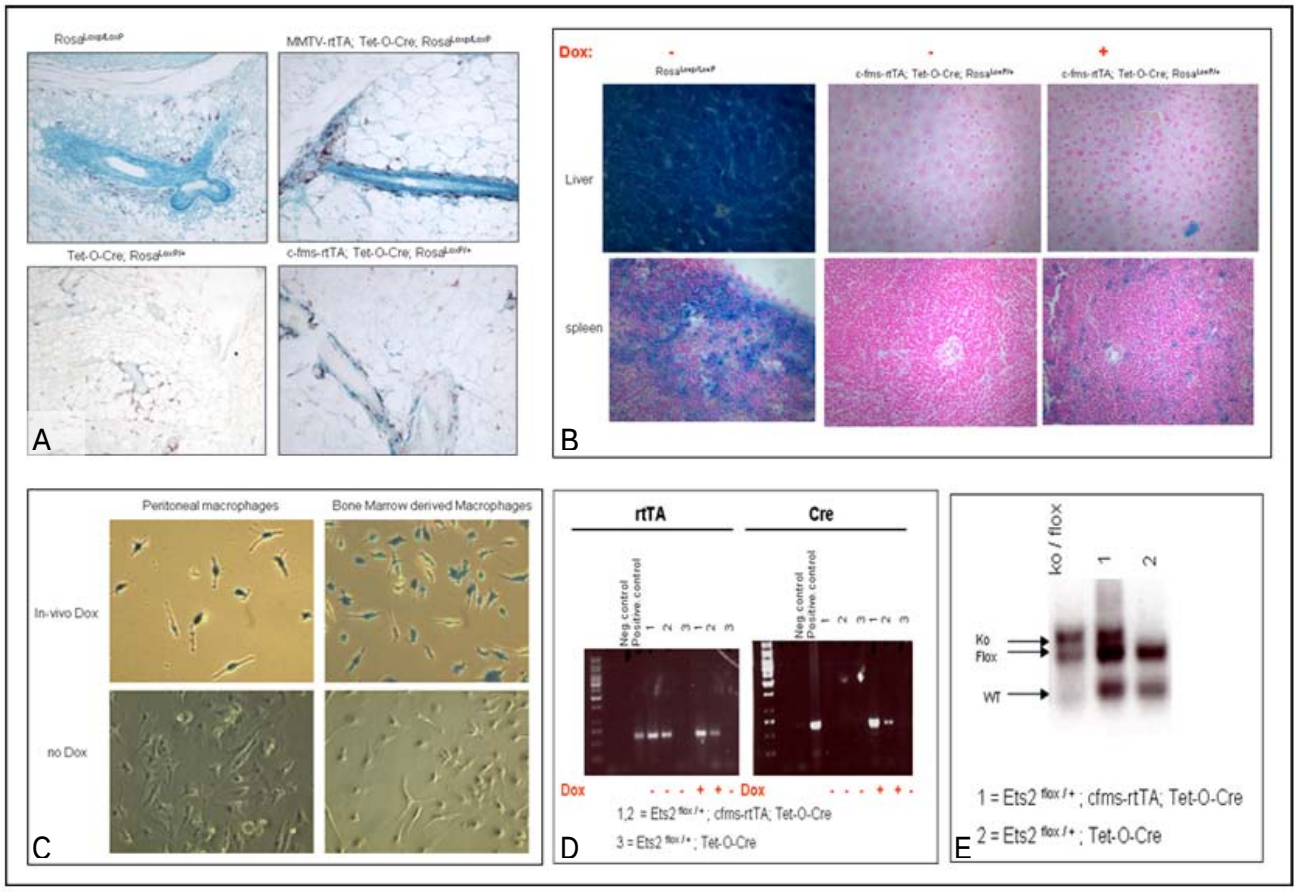

Figure 4: Characterization of one c-fms-rtTA-M2 transgenic line. A. Immuno-histochemical analysis of macrophage distribution in X-gal stained (blue) mammary glands. Rat α-mouse F4/80 antibody was used as a marker for macrophages (stained purple). Mice were fed on Dox food for ~4 weeks. In mice X-gal stained cells over-lap with F4/8 stained cells (bottom right). As a positive control,mammary gland extracted from MMTV-rtTA;tet-O-Cre;Rosa-LoxP mouse was used (expresses rtTA only in epithelial cells). B. Myeloid organs like spleen also stain positively for X-gal; whereas Kupffer cells in liver do not seem to express rtTA.. C. Macrophages extracted from peritoneum (thioglycolate elicited) and bone marrow from Dox-fed animals stain positive for X-gal whereas non-Dox fed animals do not. D. Cre is only induced in the presence of Dox-treatment as seen from mRNA extracted from cultured bone marrow derived macrophages from c-fms--rtTA;tet-O-Cre; Ets-2 f/f animals. Concommittantly, there is partial deletion of ets-2 as seen by semi-quantitative PCR analysis (E).

Key research accomplishments

- Deletion of ets-2 in specific compartments in the TME has different effects on tumor growth. This study indicates that deletion of ets-2 in the macrophages causes decreased metastatic lung lesions. Interestingly, deletion of ets-2 in the the fibroblast or the epithelial compartment leads to decreased (ongoing studies in our lab) or no effect on tumor growth respectively.
- Ets-2 might act as a repressor in the macrophage compartment of the TME.
- Generation of an inducible c-fms-rtTA transgenic line may be a useful tool to tease the temporal roleof ets-2 during tumor progression.

Reportable outcome

- Poster presentation:
  - Genetic Analysis of *ets-2* in Tumor-Associated Macrophages During Breast Cancer Progression
    Fourth International conference of Tumor Micro-environment: progression, therapy and prevention, Florence, Italy
  - Genetic Analysis of *ets-2* in Tumor-Associated Macrophages During Breast Cancer Progression
    AACR Annual Meeting, Los Angeles, USA

References

1. Banerjee, S., et al., *Mechanisms of disease: angiogenesis and the management of breast cancer*. Nat Clin Pract Oncol, 2007. **4**(9): p. 536-50.
2. Tabruyn, S.P. and A.W. Griffioen, *Molecular pathways of angiogenesis inhibition.* Biochem Biophys Res Commun, 2007. **355**(1): p. 1-5.
3. Hunter, K.W., et al., *Predisposition to efficient mammary tumor metastatic progression is linked to the breast cancer metastasis suppressor gene Brms1.* Cancer Res, 2001. **61**(24): p. 8866-72.
4. Steeg, P.S., *Metastasis suppressors alter the signal transduction of cancer cells.* Nat Rev Cancer, 2003. **3**(1): p. 55-63.
5. Lamartina, S., et al., *Stringent control of gene expression in vivo by using novel doxycycline-dependent trans-activators.* Hum Gene Ther, 2002. **13**(2): p. 199-210.